

## NOTES

### A Method for Detection of Aromatic Metabolites at Very Low Concentrations: Application to Detection of Metabolites of Anaerobic Toluene Degradation†

ELIZABETH A. EDWARDS,<sup>1\*</sup> ALED M. EDWARDS,<sup>2</sup> AND DUNJA GRBIĆ-GALIĆ<sup>1</sup>

*Environmental Engineering and Science, Department of Civil Engineering, Stanford University, Stanford, California 94305-4020,<sup>1</sup> and Department of Pathology, McMaster University, Hamilton, Ontario L8N 2Z5, Canada<sup>2</sup>*

Received 19 May 1993/Accepted 2 November 1993

**Difficulties inherent in working with anaerobic microorganisms and mixed cultures have hampered efforts to detect and identify metabolites of anaerobic degradation of monoaromatic compounds. Isotope-trapping experiments and analysis using a high-performance liquid chromatograph equipped with a flow-through radioactivity detector were used to detect very low concentrations of metabolites. Data obtained by this method suggest that toluene was degraded via methyl hydroxylation by a mixed methanogenic culture.**

Research on the anaerobic biodegradation of monoaromatic hydrocarbons, like toluene, has been hampered by difficulties associated with studying anaerobic microorganisms (low growth rates, poorly defined growth requirements, and the need for specialized equipment). The detailed biochemical analysis of anaerobic biodegradation is plagued by additional problems. Strict anaerobes found in sulfate-reducing and methanogenic cultures are inhibited by high substrate concentrations (for toluene, typically less than 400  $\mu$ M), and thus only low amounts of substrate can be used to sustain growth. As a result of these low substrate concentrations, together with the low rates of growth and degradation, and possibly the relatively small amount of energy available from the reaction (4, 5), intermediates in the catabolic pathways do not appear to accumulate and have proven very difficult to detect. A high-performance liquid chromatography (HPLC)-radioactive tracer method presented in this paper offers a sensitive way to detect metabolites at very low concentrations. Radioactive tracing and isotope trapping are very effective techniques for determining metabolic pathways, especially when the concentrations are very low, because these low concentrations can be overcome by using a radioactive substrate with high specific activity. Labeled substrates also provide an indisputable link between the substrate and any labeled products detected. Our method was applied to the search for metabolites of anaerobic toluene degradation by a mixed methanogenic culture (4). The data which we present suggest that toluene degradation by this methanogenic culture proceeded via methyl hydroxylation to benzyl alcohol, followed by further oxidation steps to benzaldehyde and benzoate, with perhaps a parallel pathway via ring hydroxylation to *p*-cresol.

We first attempted to determine intermediates of metha-

nogenic toluene degradation by direct HPLC analysis of the culture medium, but no metabolites were detected. We then conducted a simultaneous adaptation experiment (11) to suggest possible intermediates. Each of eight compounds (*p*-, *m*- and *o*-cresol; benzyl alcohol; benzaldehyde; benzoate; phenol; and methylcyclohexane), which are good candidates for intermediates, was fed simultaneously with toluene to the methanogenic culture. This experiment is based on the prediction that a true intermediate in the catabolic reaction would (i) be degraded without a lag and (ii) inhibit degradation of the substrate (i.e., toluene). In an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.), 18 16-ml glass vials were each filled with 10 ml of active methanogenic culture. The test substances (except methylcyclohexane) were added individually to these vials from aqueous stock solutions to a final concentration of 100  $\mu$ M. Methylcyclohexane (100  $\mu$ M) was added as a neat solution with a 1- $\mu$ l syringe. All chemicals were purchased from Sigma (St. Louis, Mo.) or Aldrich (Milwaukee, Wis.) and were greater than 99.9% pure. Duplicate vials were prepared per test compound. The vials were sealed with Mininert screw caps (Alltech Associates, Inc. Deerfield, Ill.) and amended with neat toluene to a final concentration of 100  $\mu$ M (0.15  $\mu$ l per vial). Two control vials were amended with toluene only. The vials were incubated at 35°C in an anaerobic chamber. The initial rates of degradation of toluene and of the added test substances were calculated from the initial concentration versus time data (first 4 days). Toluene and methylcyclohexane concentrations were measured by headspace analysis with a gas chromatograph (GC) equipped with a photoionization detector (5). The concentrations of the remaining compounds were measured by HPLC. A 0.5-ml sample was removed from the vial with a sterile, disposable syringe and placed in a 2-ml screw-cap vial. The vial was centrifuged for 15 min at 6,000  $\times g$ . The supernatant was injected onto the HPLC column (described below) via a 100- $\mu$ l sample loop. The results of the simultaneous adaptation experiment are shown in Table 1. *m*-Cresol, phenol, and methylcyclohexane were not degraded by this culture. Me-

\* Corresponding author. Present address: Beak Consultants Ltd., 42 Arrow Rd., Guelph, Ontario, Canada, N1K-1S6. Phone: 519-763-2325 x236. Fax: 519-763-2378. Electronic mail address: Edwardsa@fhs.McMaster.ca.

† Dedicated to the memory of Dunja Grbić-Galić (1950 to 1993).

TABLE 1. Effects of potential toluene-degradative intermediates

Potential substrate <sup>a</sup>	Initial rate of degradation ( $\mu\text{M/day}$ ) <sup>b</sup>	
	Toluene	Test substance
Toluene alone	10.5 $\pm$ 0.7	None added
Toluene + benzyl alcohol	2.0 $\pm$ 3.2	10.0 $\pm$ 2.0
Toluene + benzaldehyde	7.3 $\pm$ 2.9	8.8 $\pm$ 1.5
Toluene + benzoate	6.5 $\pm$ 3.1	5.7 $\pm$ 0.9
Toluene + <i>p</i> -cresol	10.3 $\pm$ 1.3	3.8 $\pm$ 3.1
Toluene + <i>o</i> -cresol	10.6 $\pm$ 1.2	0 (degraded after lag)
Toluene + <i>m</i> -cresol	9.6 $\pm$ 0.4	0
Toluene + phenol	10.7 $\pm$ 0.6	0
Toluene + methylcyclohexane	5.7 $\pm$ 4.6	0

<sup>a</sup> Toluene and the test substance were added at an initial concentration of 100  $\mu\text{M}$ .

<sup>b</sup> Data are the means of duplicates  $\pm$  standard deviations.

thylcyclohexane appeared to have a partially inhibitory effect on toluene degradation. Degradation of *o*-cresol began eventually, after a lag of about 1 week. Benzyl alcohol, benzaldehyde, benzoate, and *p*-cresol were all degraded by the culture without a lag. Benzyl alcohol was an effective inhibitor of toluene degradation (Table 1). In addition, benzoic acid was observed to transiently accumulate in cultures fed either benzyl alcohol or benzaldehyde (data not shown).

The data from the simultaneous adaptation experiments identified benzyl alcohol, benzaldehyde, benzoate, and *p*-cresol as possible intermediates. Because the simultaneous adaptation method is indirect and based on questionable assumptions, we sought a more direct method to confirm these metabolites or to identify other metabolites. To overcome the problem of the low concentrations involved, we decided to take advantage of the sensitivity of radioactive analyses, and thus a method for assaying radioactive products was sought. The initial intent was to detect radioactive metabolites by direct injection of culture fluid onto an HPLC coupled to UV and radioactivity detectors in series.

**HPLC and radioactivity detector.** The HPLC system consisted of a series 3B pump (Perkin-Elmer, Norwalk, Conn.), a Rheodyne injector (0.5 ml loop), a C<sub>18</sub> reversed-phase column (inside diameter, 250 mm by 4.6 mm) (Absorbosphere HS; Alltech), a Perkin-Elmer model 240 diode array spectrometer (UV detector), and a flow-through radioactivity detector ( $\beta$ -RAM, model 1; IN/US Systems, Inc., Tampa, Fla.). The latter was connected in series after the UV detector. The signals from the radioactivity and UV detectors were processed with software from IN/US. The UV detector was also connected to a Nelson system interface (Perkin-Elmer) and to a plotter for displaying UV spectral information. With this system, it was possible to obtain simultaneous UV and radioactive traces for the peaks eluting from the column. The UV detector also recorded the complete UV spectrum for each peak detected. Two mobile phases were used: mobile phase A was 50 mM phosphate buffer (pH 3), and mobile phase B was 100% acetonitrile. Potential metabolites and toluene were eluted from the column with the following gradient: 80% A–20% B, isocratic for 52 min, linear gradient to 35% A–65% B over a period of 20 min, and 35% A–65% B, hold for 10 min. The proportion of acetonitrile was kept below 65% to preclude precipitation of the phosphate buffer. The mobile phase flow rate was 1 ml/min. The radioactivity detector was equipped with a 1-ml liquid flow cell. Scintillation fluid (INFLO 3; IN/US Systems, Inc.) was mixed with the effluent from the UV detector

at a ratio of 3:1 before going through the radioactivity detector.

In preliminary experiments, cultures were fed radiolabeled toluene, and the culture fluid was analyzed for radiolabeled products by HPLC; no significant radioactive peaks were detected, and peaks that might elute with low retention times were swamped by a huge peak corresponding to labeled dissolved carbonate species. Furthermore, metabolites that may have remained inside or attached to cells were not recovered by this method, since cells were removed by centrifugation before the sample was injected onto the HPLC column. An ether extraction procedure that solved many of these problems was developed. By extraction into ether, the dissolved CO<sub>2</sub> peak disappeared; the cells were lysed; and large aqueous volumes could be extracted, concentrated, and injected at one time, increasing the sensitivity. Because ether is immiscible with the HPLC mobile phase, the concentrated ether extract had to be extracted back into a water-miscible phase. Methanol was added just before the last of the ether evaporated to increase extraction efficiency. The experimental details of this extraction procedure are described below. A selective extraction of nonvolatile, water-soluble compounds resulted; this category of compounds likely includes intermediates of toluene degradation. With this extraction-concentration scheme, it was necessary to begin with radiolabeled toluene free of contaminating radioactive species. The purchased labeled toluene was highly contaminated with labeled nonvolatile components. These contaminants were not only potential intermediates in the metabolism of toluene, but they were also present in concentrations representing 1 to 2% of the label in toluene. Therefore, [<sup>14</sup>C]toluene was further purified by a purge and trap method. Five hundred microcuries of toluene (1  $\mu\text{Ci}/\mu\text{mol}$ ) was transferred into 5 ml of water, to which 0.5 ml of 2 N NaOH had been added. This solution was purged with N<sub>2</sub> and trapped in a 600- $\mu\text{l}$  reacti-vial (Alltech Associates, Inc.) containing 100  $\mu\text{l}$  of methanol. The reacti-vial was cooled with an isopropanol-dry ice mixture. The toluene was purged slowly for about 1 h. Ninety percent of the toluene was recovered in the methanol, giving a final activity of about 3.9  $\mu\text{Ci}/\mu\text{l}$  of toluene-methanol mixture. The radiochemical purity of toluene was thus increased from 98% to 99.99%.

Experiments with radiolabeled toluene were conducted in a series of 40-ml vials sealed with Mininert valves. Twenty milliliters of active toluene-degrading methanogenic culture was added to each vial inside an anaerobic chamber. To add the radiolabel, the vials were removed from the anaerobic chamber and placed in a fume hood. Depending on the experiment, 2 to 19  $\mu\text{Ci}$  of purified [<sup>14</sup>C]toluene was injected into each vial through the Mininert valve with a 10- $\mu\text{l}$  syringe. The vials were immediately placed inside an anaerobic jar that was continuously flushed with N<sub>2</sub>. Mininert valves provided an excellent seal for organic compounds such as toluene but were quite leaky for gases (H<sub>2</sub>, O<sub>2</sub>, and CO<sub>2</sub>). Therefore, the vials were incubated inside an anaerobic jar in a fume hood (rather than in an anaerobic chamber). The anaerobic jar was continuously flushed with nitrogen to prevent oxygen from diffusing into the jar and into the sample vials. During the procedure of removing the vials from the anaerobic chamber, spiking them with radiolabeled toluene, and transferring them to a N<sub>2</sub>-flushed anaerobic jar, none of the vials became contaminated with oxygen (oxygen would have caused the vials to turn pink because the medium contained the redox indicator resazurin).

**Description of the extraction method for HPLC analysis of**

**radiolabeled compounds.** Vials were removed from the anaerobic jar, and the culture fluid was extracted following the steps outlined below (all steps were performed in a fume hood). For the first extraction (at neutral pH), 10 ml of diethyl ether was injected directly into the sample vial with a glass syringe equipped with a 22-gauge, 2.5-in. (ca. 6.4-cm) stainless steel needle. The septum was first removed from the Mininert valve of the sample vial to avoid a buildup of pressure. The sample vial was shaken vigorously by hand for 3 min and vented by using the Mininert valve, and the mixture was allowed to settle for about 30 min. With the same glass syringe, the ether phase was transferred to a clean 40-ml vial (containing 150  $\mu$ l of phosphate buffer [pH 3]) and capped with a Teflon-lined screw-cap. For the second extraction (at pH 2), the glass syringe was re-inserted into the sample vial (without the plunger), 400  $\mu$ l of 6 N HCl was added through the syringe barrel to acidify the sample, and then 10 ml of clean ether was added. The syringe contents were injected into the sample vial. The samples were acidified to protonate organic acids such as benzoate, thus rendering them more soluble in ether. The sample vial was shaken vigorously by hand for 3 min and vented by using the Mininert valve, and the mixture was allowed to settle for about 30 min (the pH was monitored with pH paper). Again, with the glass syringe, the second ether phase was transferred to the 40-ml vial containing the first ether phase and capped. A final 10 ml of diethyl ether was added to the sample vial, and a third extraction was completed as described for the second extraction. The combined ether extracts totaled about 30 ml. The combined ether extracts were evaporated under a stream of nitrogen while the vial was immersed in a warm water bath (35°C). Since the vial originally contained 150  $\mu$ l of phosphate buffer and accumulated water from the ether, the final aqueous volume was around 200  $\mu$ l. When the volume of ether left was less than the aqueous volume (as determined by visual inspection), 100  $\mu$ l of methanol was added to the sample, and then the remaining ether was completely evaporated. The remaining aqueous extract (approximately 300  $\mu$ l) thus recovered was ready to be injected onto the HPLC column. Samples containing particulate material were clarified by centrifugation before injection. Typically, about half the sample was injected onto the HPLC column, and the other half was saved for possible analysis by gas chromatography-mass spectrometry. Initially, various surrogate standards were added to the vials to monitor the extractions; however, it was found that the resazurin (or a reduction product of resazurin) present in the medium eluted at a convenient retention time in the UV chromatogram and could serve as a surrogate standard. On the basis of extractions with standard solutions of benzoate, benzaldehyde, cresols, phenols, and reduced resazurin, the extraction efficiency ranged from 38 to 55%.

**Detection limit.** The sensitivity of the radioactivity detector depends, in part, on the size of the flow cell: the larger the cell, the higher the sensitivity, but the lower the resolution. With a 1-ml flow cell (as used in our experiments), a peak of 150 cpm or 2.5 Bq (about five times noise level) could be detected. On the basis of this detection limit and a substrate specific activity of 1  $\mu$ Ci/ $\mu$ mol, we could detect labeled compounds as dilute as 10 nM in a 20-ml culture fluid; this is comparable to the detection limits of gas chromatography-mass spectrometry. The sensitivity could be increased further by using a substrate with higher specific activity.

Even with the sensitivity of the overall extraction-HPLC

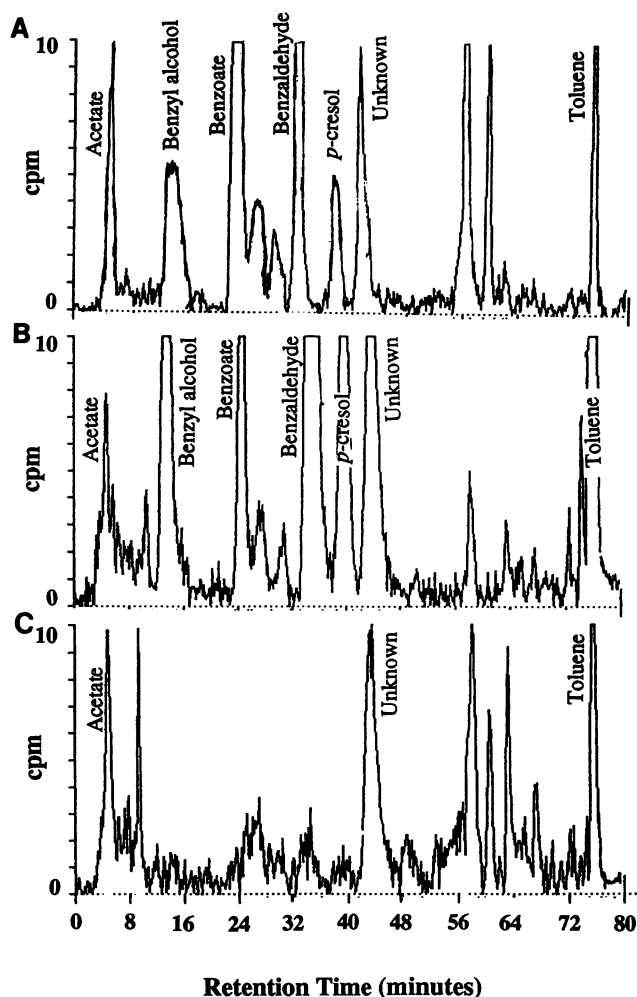


FIG. 1. Three chromatograms from HPLC analysis showing radioactivity detector output from methanogenic culture amended with radiolabeled toluene and unlabeled benzoate (A), with radiolabeled toluene and unlabeled benzyl alcohol (B), and with radiolabeled toluene only (C).

method, we did not detect any significant radioactive products in cultures fed labeled toluene, even after increasing the amount of [ $^{14}$ C]toluene added per vial from 2 to 19  $\mu$ Ci. This indicated that metabolites did not normally accumulate to concentrations higher than the detection limit of 10 nM. To increase the concentration of labeled metabolites, we applied the method of isotope trapping. Cultures actively degrading labeled toluene were challenged with a large dose of an unlabeled compound (i.e., a potential intermediate) to trap radiolabel in upstream metabolites. Six 40-ml glass vials filled with 20 ml of active methanogenic culture were spiked with 19  $\mu$ Ci of labeled toluene (5  $\mu$ l of purified labeled toluene-methanol mixture per vial). After 3 days of incubation, approximately 35% of the toluene was degraded (as determined by liquid scintillation counting of a small aliquot from a vial). At this time, five vials were quickly removed from the anaerobic jar and spiked with 200  $\mu$ M of either benzyl alcohol, benzoate, *p*-cresol, *m*-cresol or *o*-cresol. The sixth vial was not amended. Four days later, samples from all the vials were extracted and analyzed.

Figure 1 shows the chromatograms obtained from the

TABLE 2. Radioactivity recovered in toluene metabolites after addition of isotope-trapping compounds to a [ $^{14}\text{C}$ ]toluene-degrading methanogenic culture

Radiolabeled metabolite <sup>a</sup>	Retention time (min)	Radioactivity recovered (cpm) <sup>b</sup> after addition of unlabeled isotope-trapping compounds					
		None	Benzyl alcohol	Benzoate	<i>p</i> -Cresol	<i>o</i> -Cresol	<i>m</i> -Cresol
Benzyl alcohol	13.4	0	1,590	480	160	0	0
Benzaldehyde	32.7	0	4,730	1,040	0	0	0
Benzoate	23.5	0	1,090	5,200	100	0	0
<i>p</i> -Cresol	38.5	0	1,620	350	590	250	320
Unknown	42.8	1,282	1,860	800	1,020	200	230

<sup>a</sup> Metabolite corresponding to the HPLC peak.<sup>b</sup> In these experiments, 150 cpm corresponded to approximately 10 nM.

radioactivity detector after HPLC analysis of samples from three vials from the isotope trapping experiment: one that had been amended with benzoate as a trapping agent, one that had been amended with benzyl alcohol, and finally one that had no amendments. All the radioactivity recovered in the various peaks originated from labeled toluene. Peaks of radioactivity corresponding to benzoate, benzyl alcohol, benzaldehyde, *p*-cresol, toluene, and acetate were identified by matching retention times and UV spectra with those of authentic standards (Fig. 1). Authentic standards of many potential intermediates, including *p*-hydroxybenzoate, *p*- and *o*-toluate, *m*- and *o*-cresol, cyclohexane carboxylic acid, phthalate, phenylacetate, and phenylpropionate, were analyzed for comparison with unknown peaks. None of these compounds corresponded to the unknown peaks of the chromatograms. The amount of radioactivity found in each peak (normalized on the basis of the area of the resazurin peak in the UV chromatogram) is shown in Table 2. When no trapping agent was added to the culture, very few peaks could be detected in the region of interest (i.e., the region spanning retention times from 9 to 50 min, at which all standards of potential aromatic intermediates eluted). Similarly, *o*- and *m*-cresol did not cause a significant buildup of radioactive metabolites (Table 2). However, benzyl alcohol and benzoate were very effective trapping agents. Both compounds caused significant increases in the radioactivity detected in the peaks corresponding to benzyl alcohol, benzaldehyde, benzoate, and *p*-cresol (Table 2). *p*-Cresol was less effective, causing a barely detectable buildup of benzyl alcohol and a small accumulation of labeled *p*-cresol. An unidentified radiolabeled peak eluting at about 42.8 min was detected in all the samples; it appeared to accumulate in the culture fluid.

The combination of isotope trapping and radiodetection used in this study has enabled the identification of several compounds as possible intermediates in the methanogenic degradation of toluene. Some or all of the compounds identified, namely benzoic acid, benzyl alcohol, benzaldehyde, and *p*-cresol, have previously been implicated in anaerobic toluene degradation (1–3, 6–10, 12). To determine the metabolic relationship between the various intermediates, to distinguish between major and minor pathways of degradation, and to identify possible dead-end metabolites, the accumulation of label in the various compounds during isotope trapping experiments must be determined as a function of time, perhaps by using a pulse-chase procedure.

To test the general applicability of our method, we used this radioactivity detection system to study the degradation of radiolabeled toluene in a sulfate-reducing toluene-degrading culture (5). To our surprise, even in the absence of trapping compounds, several intermediates were detected.

Though most of the intermediates remain uncharacterized at present (only benzoate and benzylsuccinate have been identified [3a]), their abundance was 50- to 100-fold higher than the abundance of those observed in the methanogenic culture. These studies indicate that there are significant variations between cultures in the relative accumulation of the catabolic intermediates and therefore suggest that a judicious choice of the culture may greatly aid in the identification of the elusive initial anaerobic biotransformation intermediates.

This project was supported through the U.S. Environmental Protection Agency-supported Western Region Hazardous Substance Research Center at Stanford University, grants from the U.S. Air Force (AFOSR 88-0351), U.S. Environmental Protection Agency (EPA R 815252-01-0), and NSF (NSF CES 8813958) awarded to D.G.-G. and a scholarship from the Quebec government (F.C.A.R.) awarded to E.A.E. A.M.E. is a Research Scholar of the Medical Research Council of Canada.

We thank Harry Beller, Harry Ball, and Ned Black for valuable discussions and two anonymous reviewers for critical review of the manuscript.

## REFERENCES

- Altenschmidt, U., and G. Fuchs. 1991. Anaerobic degradation of toluene in denitrifying *Pseudomonas* sp.: indication for toluene methylhydroxylation and benzoyl-CoA as central aromatic intermediate. *Arch. Microbiol.* **156**:152–158.
- Beller, H. R., M. Reinhard, and D. Grbić-Galić. 1992. Metabolic by-products of anaerobic toluene degradation by sulfate-reducing enrichment cultures. *Appl. Environ. Microbiol.* **58**:3192–3195.
- Dolfing, J., J. Zeyer, P. Blinder-Eicher, and R. P. Schwarzenbach. 1990. Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. *Arch. Microbiol.* **154**:336–341.
- Edwards, E. Unpublished data.
- Edwards, E. A., and D. Grbić-Galić. 1994. Anaerobic biodegradation of toluene and *o*-xylene by a methanogenic consortium. *Appl. Environ. Microbiol.* **60**:313–322.
- Edwards, E. A., L. E. Wills, M. Reinhard, and D. Grbić-Galić. 1992. Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. *Appl. Environ. Microbiol.* **58**:794–800.
- Evans, P. J., W. Ling, B. Goldschmidt, E. R. Ritter, and L. Y. Young. 1992. Metabolites formed during anaerobic transformation of toluene and *o*-xylene and their proposed relationship to the initial steps of toluene mineralization. *Appl. Environ. Microbiol.* **58**:496–501.
- Grbić-Galić, D., and T. M. Vogel. 1987. Transformation of toluene and benzene by mixed methanogenic cultures. *Appl. Environ. Microbiol.* **53**:254–260.
- Kuhn, E. P., J. Zeyer, P. Eicher, and R. P. Schwarzenbach.

1988. Anaerobic degradation of alkylated benzenes in denitrifying laboratory aquifer columns. *Appl. Environ. Microbiol.* **54**: 490–496.
9. **Lovley, D. R., and D. J. Lonergan.** 1990. Anaerobic oxidation of toluene, phenol and *p*-cresol by the dissimilatory iron-reducing organisms GS-15. *Appl. Env. Microbiol.* **56**:1858–1864.
10. **Schocher, R. J., B. Seyfried, F. Vasquez, and J. Zeyer.** 1991. Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. *Arch. Microbiol.* **157**:7–12.
11. **Stanier, R. Y.** 1947. Simultaneous adaptation: a new technique for the study of metabolic pathways. *J. Bacteriol.* **54**:339–348.
12. **Vogel, T. M., and D. Grbić-Galić.** 1986. Incorporation of water into toluene and benzene during anaerobic fermentative transformation. *Appl. Environ. Microbiol.* **52**:200–202.